Role of local muscle contractile activity in the exercise-induced increase in NR4A receptor mRNA expression

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Submitted 18 July 2008; accepted in final form 23 March 2009

Kawanaka K, Hokari F, Sasaki M, Sakai A, Koshinaka K, Kawamura K. Role of local muscle contractile activity in the exercise-induced increase in NR4A receptor mRNA expression. J Appl Physiol 106: 1826–1831, 2009. First published April 9, 2009; doi:10.1152/japplphysiol.90923.2008.—Exercise upregulates the expression of NR4A receptors, which are involved in regulation of glucose and fatty acid utilization genes in skeletal muscle. The aims of our study were 1) to determine the role of contractile activity on NR4A mRNA expression in skeletal muscle during exercise; and 2) to elucidate the mechanisms underlying the induction of NR4A mRNA expression in response to muscle contractile activity. Rats were subjected to an acute 3-h low-intensity swimming or a 3-h low-intensity treadmill running as a model of endurance exercise. Low-intensity swimming increased NR4A1 and NR4A3 mRNA in triceps but not in soleus muscle. Conversely, low-intensity treadmill running increased NR4A1 and NR4A3 mRNA in soleus but not in triceps muscle. NR4A mRNA increased concomitantly with reduced postexercise muscle glycogen, suggesting that gene expression of NR4A receptors occurs in muscles recruited during exercise. Furthermore, in resting rats, an acute 1-h local electrical stimulation of a motor nerve to the tibialis anterior muscle caused increases in NR4A1 and NR4A3 mRNA relative to the contralateral control muscle of the same animals. On the other hand, after 6 h of hindlimb immobilization, NR4A1 and NR4A3 mRNA were reduced in immobilized soleus muscle relative to contralateral control muscle. In addition, both NR4A1 and NR4A3 mRNA in epimysciar muscle were increased after 6-h incubation with 0.5 mM 5-aminoimidazole-4-carboxamide-1β-D-ribofuranoside, which activates AMP-activated protein kinase. These results suggest that 1) local muscle contractile activity is required for increased expressions of NR4A1 and NR4A3 mRNA during exercise; and 2) muscle contractile activity–induced increases in NR4A1 and NR4A3 mRNA may be mediated by AMPK activation, at least in part.

swimming; treadmill running; Nur77; NOR1; electrical stimulation; immobilization; adenosine 5′-monophosphate-activated protein kinase

SKELETAL MUSCLE IS A MAJOR mass metabolic tissue that accounts for ∼40% of the total body weight. This peripheral lean tissue is the primary site for fatty acid oxidation. Furthermore, this tissue is the main target of insulin-stimulated glucose uptake and accounts for ∼80% of glucose disposal (4). Therefore, the ability of skeletal muscle to utilize glucose and fatty acid plays a role in the pathophysiological progression of metabolic diseases, such as dyslipidemia, diabetes, and obesity. On the other hand, physical exercise increases the ability of muscle to utilize glucose and fatty acid, leading to the prevention of metabolic diseases (1, 5, 15). However, the molecular regulatory mechanism(s) responsible for such exercise-induced muscle adaptations has not been fully understood.

The NR4A orphan nuclear receptor subfamily consists of three well-conserved members, NR4A1, NR4A2, and NR4A3, otherwise known in mouse as Nur77, Nurr1, and NOR1, respectively. Although the potential roles of NR4A receptors in metabolism have not been well explored, previous studies have demonstrated that attenuation of NR4A1 or NR4A3 gene expression reduced the expression of genes associated with glucose and fatty acid utilization [e.g., glucose transporter (GLUT)-4, fatty acid translocase/CD36, peroxisome proliferator-activated receptor-α/β coactivator-1, lipin-1α, etc.] in skeletal muscle cell line (14, 18, 19). Furthermore, Fu et al. (6) reported that gene expressions of NR4A1 and NR4A3 were reduced in skeletal muscles of diabetic and insulin-resistant animals. They also showed that hyperegpression of NR4A3 increased the ability of insulin to stimulate glucose transport and GLUT-4 translocation. Thus NR4A1 and NR4A3 gene expression are thought to be involved in the regulation of genes that control glucose and fatty acid utilization in skeletal muscles.

A previous study showed that expressions of NR4A receptor mRNA were upregulated in vastus lateralis muscle in human subjects 3 h after endurance exercise to exhaustion (12), suggesting the possibility that increased gene expression of NR4A receptor is a potential mediator of exercise-induced enhancement of glucose and fatty acid utilization. Although we do not know specifically how muscle senses “exercise signals” to induce NR4A gene expression, gene expression of NR4As has been reported to be increased by β-adrenergic receptor agonists in skeletal muscle (14, 18, 19). Since β-adrenergic receptors and their agonists mediate the action of the sympathetic nervous system (SNS), which innervates skeletal muscles, SNS activity is an important systemic factor that may regulate NR4A gene expression in skeletal muscle. Furthermore, since exercise causes marked activation of the SNS (3), it is possible that exercise-induced NR4A gene expression in skeletal muscle is due to activation of the SNS. In contrast, exercise-induced adaptive responses in skeletal muscle metabolism, such as mitochondrial biogenesis, are mostly mediated by local factors in contracting muscle rather than systemic factors (8). In addition, there is considerable evidence that activation of AMP-activated protein kinase (AMPK) mediates exercise-induced local adaptations in skeletal muscle through increase in AMP and decreases in phosphocreatine and ATP that occur in contracting muscles (7, 22). However, the role of local contractile activity and AMPK activation on NR4A gene expression during exercise is currently unknown.

To test the role of local contractile activity on NR4A receptor mRNA expression during exercise in skeletal muscle, we examined whether low-intensity prolonged exercise, a

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model of endurance exercise, increases NR4A receptor mRNA expression specifically in local contracting skeletal muscles. Furthermore, we determined the effect of local electrical stimulation and immobilization to determine whether local contractile activity is a sufficient stimulus to regulate NR4A mRNA expression. In addition, we examined whether activation of AMPK, which occurs in contracting muscles during exercise, induces increases in NR4A mRNA expression in skeletal muscles. Finally, because it is generally believed that endurance and sprint-type exercise induce different adaptations in skeletal muscle, we examined the effects of high-intensity intermittent exercise, a model of sprint interval exercise, on NR4A mRNA expression in skeletal muscle, and compared them with the effects of low-intensity prolonged exercise.

MATERIALS AND METHODS

Treatment of Animals

This research was approved by the Animal Studies Committee of Niigata University of Health and Welfare. Male Wistar rats were obtained from CLEA Japan (Tokyo, Japan). Animals were maintained in individual cages and fed a standard rodent chow diet and water ad libitum.

Endurance Exercise Protocol of Experiment 1

Two days before the experiment, all rats were acclimated to swimming or treadmill running for 10 min. Rats (100–120 g) were divided into a resting control (Rest) group, a low-intensity prolonged swimming (LIS) group, or a low-intensity prolonged treadmill running (LIR) group. All rats were fed ad libitum until the LIS or LIR group started exercise. When the LIS or LIR group rats started exercise, Rest group rats started fasting. Rats in the LIS group swam for 3 h with a weight (11). Water temperature was maintained at 35°C during the swimming. Rats in the LIR group ran on a motorized treadmill up a 15% grade at 9 m/min for 3 h.

Following the LIS exercise, rats were killed at 8:00 PM by cervical dislocation immediately after completion of the exercise, and triceps and soleus muscles were dissected and clamp-frozen in liquid nitrogen for measurement of glycogen concentration, acetyl-CoA carboxylase (ACC) phosphorylation, and NR4A mRNA. Following the LIR exercise, rats were killed at 8:00 PM by cervical dislocation immediately after completion of the exercise, and triceps and soleus muscles were dissected and clamp-frozen in liquid nitrogen for measurement of glycogen concentration, ACC phosphorylation, and NR4A mRNA.

The LIS and LIR groups were time matched with the Rest group, with the tissues of the Rest group rats being collected at the same time as those of the exercised rats.

Immobilization Protocol of Experiment 2

For the second set of experiments, rats (100–120 g) were subjected to unilateral hindlimb immobilization. Plantar cast (Castlight, ALCARE, Tokyo, Japan) was applied to the left hindlimb of rats without anesthetization. The leg was immobilized at the plantar flexion position. Results from pilot studies (data not shown) indicated that unilateral immobilization had no effect on various parameters in skeletal muscle of the contralateral noncasted leg. Consequently, the contralateral hindlimb served as the control in our experiment. After casting, rats were housed individually. Immobilization was imposed for 6 h. All rats were maintained in individual cages and fed a standard rodent chow diet and water ad libitum until the muscle sampling. For muscle sampling, casts were removed under pentobarbital sodium anesthesia (5 mg/100 g body wt), and soleus muscles from both the immobilized and contralateral hindlimb were dissected and clamp-frozen in liquid nitrogen for subsequent measurement of NR4A mRNA and protein.

Electrical Stimulation Protocol of Experiment 3

Rats were anesthetized with pentobarbital sodium (1.5 mg/100 g body wt). To induce contractile activity, the tibialis anterior muscle was stimulated indirectly using subminiature electrodes (Harvard Apparatus, South Natick, MA) attached to the sciatic nerve 2 cm proximal to the popliteal space. SEN-3301 electrical stimulator (Nihon Kohden, Tokyo, Japan) was used to deliver supramaximal square-wave pulses of 0.1-ms duration. The leg was immobilized at the plantar flexion position. The muscles were stimulated with 250-ms trains at 100 Hz at a rate of 60 trains/min for six 9-min intervals, separated by a 1-min rest period. Muscles were dissected and clamp-frozen in liquid nitrogen for measurement of NR4A mRNA expression 3 h after cessation of contractions. Rats remained asleep during and after contractions. The nonstimulated resting control tibialis anterior muscle was obtained from the contralateral leg.

Muscle Incubation Protocol of Experiment 4

Rats were anesthetized with pentobarbital sodium (1.5 mg/100 g body wt), and the epimysialleirs muscles were removed. Epimysialleirs muscles were incubated as described previously (10). Briefly, muscles of sedentary rats were placed in 3 ml of oxygenated Krebs-Henseleit bicarbonate buffer in the presence of 8 mM glucose and 32 mM mannitol, with or without 0.5 mM 5-aminimidazole-4-carboxamide-1-β-d-ribosuramid (AICAR). The muscles were incubated for 6 h with shaking at 35°C, and the flasks were gassed continuously with 95% O2, 5% CO2. During a 6-h-long incubation, the muscles were placed in fresh incubation medium after 3 h. After incubation, some muscles were blotted and used for measurement of NR4A mRNA expression.

Sprint Exercise Protocol of Experiment 5

Two days before the experiment, all rats were acclimated to swimming for 10 min. Rats (100–120 g) were divided into a Rest group or a high-intensity short-term swimming (HIS) group. Rats in the HIS group underwent ten 20-s bouts of swimming with a weight equal to 18% body wt, with 40-s rest between bouts (11). All rats started fasting 3 h before starting swimming.

Following the exercise protocol, rats were killed at 8:00 PM by cervical dislocation immediately, 4 h, or 8 h after completion of the exercise. In animals that were killed immediately after exercise, triceps muscles were dissected and clamp-frozen in liquid nitrogen for measurement of glycogen concentration and ACC phosphorylation and NR4A mRNA expression. Animals to be killed 4 or 8 h after exercise were returned to their cages after completion of exercise and kept fasting for 4 or 8 h. The triceps muscles were then dissected out and clamp-frozen in liquid nitrogen for mRNA analysis.

The HIS group was time-matched with the Rest group, with the tissues of the Rest group rats being collected at the same time as those of the exercised rats.

Glycogen Concentration

Muscles were weighed and homogenized in 0.3 M perchloric acid, and extracts were assayed for glycogen by the amyloglucosidase method (17).

Western Blot Analysis

Triceps or soleus muscles were homogenized in ice-cold buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EDTA, 10 mM Na3PO4, 100 mM NaF, 2 mM Na3VO4, 2 mM PMSF, aprotinin (10 μg/ml), leupeptin (10 μg/ml), and pepstatin (5 μg/ml) (13). The homogenates were then rotated end over end at 4°C for 60 min and centrifuged at
Using an unpaired Student’s t-test. In experiment 5, differences were determined using a one-way analysis of variance with a subsequent Fisher’s least significant difference method. Differences between groups were considered statistically significant when \( P < 0.05 \).

RESULTS

Experiment 1: Endurance Exercise Experiment

Glycogen concentration immediately after exercise. We measured muscle glycogen concentration in skeletal muscles immediately after low-intensity prolonged exercise. As shown in Table 1, LIS exercise significantly reduced the muscle glycogen concentration in triceps muscle compared with time-matched Rest (\( P < 0.05 \)), but no difference in muscle glycogen concentration was seen in soleus muscle immediately after the cessation of LIS exercise. There was no significant change in muscle glycogen concentration in triceps muscle after LIS exercise (Table 1). However, LIR exercise did significantly decrease muscle glycogen concentration in soleus muscle immediately after exercise compared with Rest (\( P < 0.05 \)). Increased expression of NR4A1 mRNA in soleus muscle immediately after exercise compared with Rest (\( P < 0.05 \)). Neither LIS nor LIR affected total ACC in both triceps and soleus muscles.

<table>
<thead>
<tr>
<th>Muscle Glycogen Concentration, ( \mu \text{mol/g muscle} )</th>
<th>Triceps</th>
<th>Soleus</th>
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<tbody>
<tr>
<td>Rest</td>
<td>25.4 ± 0.9</td>
<td>16.0 ± 0.6</td>
</tr>
<tr>
<td>LIS</td>
<td>13.9 ± 1.5*</td>
<td>17.4 ± 1.5</td>
</tr>
<tr>
<td>LIR</td>
<td>24.7 ± 1.9</td>
<td>16.3 ± 0.6</td>
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Values are means ± SE for \( n = 6 \) muscles/group. Rest, time-matched resting control; LIS, low-intensity prolonged swimming; LIR, low-intensity prolonged treadmill running. *\( P < 0.05 \) vs. Rest.

Increased ACC phosphorylation in triceps muscle compared with time-matched Rest [Rest: 1.00 ± 0.16 arbitrary units (AU) (\( n = 8 \)), LIS: 1.93 ± 0.14 AU (\( n = 6 \)) \( P < 0.05 \)], but not in soleus muscle [Rest: 1.00 ± 0.07 AU (\( n = 4 \)), LIS: 0.86 ± 0.12 AU (\( n = 4 \))] immediately after the cessation of exercise. There was no significant change in ACC phosphorylation in triceps muscle after LIR exercise [Rest: 1.00 ± 0.12 AU (\( n = 8 \)), LIR: 0.91 ± 0.02 AU (\( n = 8 \))]. However, LIR exercise significantly increased ACC phosphorylation in soleus muscle immediately after exercise compared with Rest [Rest: 1.00 ± 0.13 AU (\( n = 7 \)), LIR: 2.77 ± 0.11 AU (\( n = 8 \)) \( P < 0.05 \)]. Neither LIS nor LIR affected total ACC in both triceps and soleus muscles.

NR4A receptor mRNA expression. As shown in Fig. 1A, LIS exercise increased NR4A1 and NR4A3 mRNA expression in triceps muscle immediately after the cessation of exercise by 3.6- and 8.3-fold, respectively, compared with time-matched Rest (\( P < 0.05 \)). The elevated expression of NR4A1 mRNA in triceps muscle returned to Rest level 4 h after the cessation of LIS exercise (data not shown). As shown in Fig. 1B, LIS exercise did not significantly change NR4A1 mRNA expression in soleus muscle immediately after exercise compared with Rest. But NR4A3 mRNA in soleus muscle decreased 67% immediately after the cessation of LIS exercise compared with the Rest group (Fig. 1B, \( P < 0.05 \)).

No significant increase in NR4A receptor mRNA was observed in triceps muscle after LIR exercise (Fig. 1C). As shown in Fig. 1D, LIR exercise increased NR4A1 and NR4A3 mRNA expression in soleus muscle immediately after the cessation of exercise by 3.0- and 30.9-fold, respectively, compared with Rest (\( P < 0.05 \)). Increased expression of NR4A1 mRNA in the soleus muscles returned to the Rest level 4 h after cessation of LIR exercise (data not shown).

Experiment 2: Immobilization Experiment

NR4A receptor mRNA expression. As shown in Fig. 2, after 6 h of immobilization, NR4A1 and NR4A3 mRNA expression was reduced in immobilized soleus muscle by 47 and 37%, respectively, relative to contralateral, nonimmobilized control muscles (\( P < 0.05 \)).

NR4A3 protein expression. NR4A3 protein expression was decreased in immobilized soleus muscle by 31% relative to contralateral, nonimmobilized control muscle [contralateral: 1.00 ± 0.04 AU (\( n = 8 \)), immobilized: 0.69 ± 0.06 AU (\( n = 8 \))] \( P < 0.05 \). Thus immobilization-induced change in...
NR4A3 mRNA expression is translated to the change in protein expression.

Experiment 3: Electrical Stimulation Experiment

NR4A receptor mRNA expression. As shown in Fig. 3, NR4A1 and NR4A3 mRNA expression was increased in electrically stimulated tibialis anterior muscle by 74 and 143%, respectively, relative to contralateral, nonstimulated control muscle (P < 0.05).

Experiment 4: AICAR Experiment

NR4A receptor mRNA expression. As shown in Fig. 4, incubation of rat epitrochlearis muscle for 6 h with 0.5 mM AICAR induced 2.8- and 3.2-fold increases in NR4A1 and NR4A3 mRNA expression, respectively, compared with basal control muscles (P < 0.05).

Experiment 5: Sprint Exercise Experiment

Glycogen concentration and ACC phosphorylation immediately after exercise. As shown in Table 2, HIS significantly decreased glycogen concentration in triceps muscle (P < 0.05) compared with Rest. On the other hand, HIS significantly increased ACC phosphorylation in triceps muscle (P < 0.05) compared with Rest [Rest: 1.00 ± 0.11 AU (n = 6), HIS: 3.14 ± 0.15 AU (n = 4), P < 0.05]. HIS did not affect total ACC in triceps muscles.

NR4A receptor mRNA expression. HIS exercise did not affect NR4A1 mRNA expression in triceps muscle (Fig. 5A). NR4A3 mRNA in triceps muscle was not increased immediately after HIS exercise, but did increase by 7.9-fold at 4 h after HIS exercise compared with time-matched Rest (P < 0.05, Fig. 5B). Increased NR4A3 mRNA expression in triceps muscle seen following HIS exercise returned to Rest level at 8 h after the cessation of exercise (Fig. 5B).
DISCUSSION

We did not know specifically how muscle senses “exercise signals” to increase NR4A receptor mRNA expression. The two general categories of stimuli are “local factors”, related to contractile activity, and “systemic factors”, related to plasma hormones and the SNS. In experiment 1 of our present study, we have shown that an acute bout of 3 h of LIR increased NR4A1 and NR4A3 mRNA expression in rat triceps muscle, but not in soleus muscle (Fig. 1, A and B). In contrast, 3 h of LIR increased mRNA expression of these genes in soleus muscle, but not in triceps muscle (Fig. 1, C and D). Finally, our results showed that NR4A1 and NR4A3 mRNA expression increased in muscles where muscle glycogen levels were reduced during exercise, but did not increase in muscles where glycogen levels are unchanged during exercise (Table 1). These results suggest that exercise-induced increases in NR4A mRNA expression are mediated by local factors intrinsic to the muscles involved in the exercise, i.e., those muscle fibers that are recruited, rather than by systemic factors. Hence, exercise-induced increases in NR4A1 and NR4A3 mRNA expression are mainly due to muscle contractile activity.

Previous study has shown that denervation of rat skeletal muscle reduced NR4A1 mRNA expression in rat skeletal muscle, demonstrating that innervation is an important factor for regulating NR4A gene expression (2). Since not only muscle contractile activity but also neural trophic factor(s) transport and/or sympathetic nervous activity are eliminated by denervation, decreased NR4A mRNA level in denervated muscle does not necessarily show that muscle contractile activity is a regulator of NR4A gene expression in skeletal muscles. Thus the role of local muscle contractile activity as a regulator of NR4A gene expression had not been reported. However, in experiment 2 of our present study, immobilization of one hindlimb, which eliminates contractile activity, lowered the levels of NR4A mRNA and protein in soleus muscle compared with contralateral, nonimmobilized muscle (Fig. 2). This result supports the idea that contractile activity is a regulator of NR4A gene expression in skeletal muscle. Further strong support for contractile activity as a regulator of NR4A gene expression in skeletal muscles comes from the result of the electrical stimulation experiment (experiment 3) presented here. Specifically, electrical stimulation of one hindlimb, which increases contractile activity, elevated the levels of NR4A1 and NR4A3 mRNA in tibialis anterior muscle compared with contralateral, nonstimulated muscle (Fig. 3).

Next, to investigate the cellular regulatory mechanism-related, exercise-induced NR4A mRNA expression, we examined the effect of AICAR, a pharmacological AMPK activator, on the NR4A mRNA expression. This is because activation of AMPK is suggested to be involved in the exercise-induced local adaptations, such as increase in GLUT-4, peroxisome proliferator-activated receptor-α coactivator-1, and mitochondrial biogenesis, in skeletal muscle through increase in AMP and decreases in phosphocreatine and ATP that occur in

Table 2. Muscle glycogen concentration in rat triceps muscle immediately after an acute bout of high-intensity short-term swimming

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<th>Muscle Glycogen Concentration, μmol/g muscle</th>
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<tbody>
<tr>
<td>Rest</td>
<td>24.1±1.8</td>
</tr>
<tr>
<td>HIS</td>
<td>5.1±0.2*</td>
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Values are means ± SE for n = 4 muscles/group. HIS, high-intensity short-term swimming. *P < 0.05 vs. Rest.
contracting muscles (9, 16, 20, 21). In experiment 4 of our present study, both NR4A1 and NR4A3 mRNA expression were increased in isolated skeletal muscle incubated for 6 h with AICAR, a pharmacological AMPK activator (Fig. 4). In addition, in experiment 1 of our study, we demonstrated that exercise increased ACC phosphorylation, an indicator of in vivo AMPK activation level, in muscles recruited during exercise (see RESULTS). Therefore, our results suggest that the increases in NR4A1 and NR4A3 mRNA expression in exercising muscles are possibly mediated by AMPK activation, at least in part.

In experiment 5 of this study, we have demonstrated that HIS, as a model of sprint interval exercise, did not increase NR4A1, but NR4A3, mRNA expression in rat triceps muscle. Since the result of our experiment 1 showed that LIS, as a model of endurance exercise, increased both NR4A1 and NR4A3 mRNA expression in triceps muscle, our present result suggests that increased gene expression of NR4A1 might be associated with specific adaptations of skeletal muscles to prolonged endurance exercise.

Previous studies have demonstrated that gene expressions of NR4A1 and NR4A3 are suppressed in skeletal muscles from rodent models of insulin resistance or diabetes (6). Moreover, they showed that overexpression of NR4A3 markedly augmented insulin responsiveness for stimulation of glucose transport and GLUT-4 translocation in adipocytes (6). These results suggest the possibility that exercise increases insulin-stimulated glucose utilization in skeletal muscle via increased expression of NR4A gene, leading to the promotion of glycogen synthesis in skeletal muscles, which are recruited during exercise. However, we have to wait until measuring NR4A protein level before concluding this possibility.

In summary, the results of this study suggest that contractile activity is a regulator of NR4A1 and NR4A3 mRNA expression in skeletal muscle, and local muscle contractile activity is required for increased expressions of NR4A1 and NR4A3 mRNA during exercise. Furthermore, we showed that exercise activates AMPK in exercising muscles and that pharmacological activation of AMPK increases NR4A receptor mRNA expression in skeletal muscle. This result suggests that the increases in NR4A1 and NR4A3 mRNA expression in exercising muscles are possibly mediated by AMPK activation, at least in part.

GRANTS

This research was supported by the Nakatomi Foundation (Tosu, Japan), a Grant-in-Aid from Niigata University of Health and Welfare, and a Grant-in-Aid for Scientific Research (KAKENHI) (C) No. 18500518 from the Japan Society for the Promotion of Science. K. Koshinaka was supported by postdoctoral fellowships from the Japan Society for the Promotion of Science.

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